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Quantitative and qualitative iNKT repertoire associations with disease susceptibility and outcome in macaque tuberculosis infection

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Abstract

Correlates of immune protection that reliably predict vaccine efficacy against *Mycobacterium tuberculosis* (Mtb) infection are urgently needed. Invariant NKT cells (iNKTs) are CD1d-dependent innate T cells that augment host antimicrobial immunity through production of cytokines, including interferon (IFN)- γ and tumour necrosis factor (TNF)- α . We determined peripheral blood iNKT numbers, their proliferative responses and iNKT subset proportions after *in vitro* antigen expansion by α -galactosylceramide (α GC) in a large cohort of mycobacteria-naïve non-human primates, and macaques from Bacillus Calmette-Guerin (BCG) vaccine and Mtb challenge studies. Animals studied included four genetically distinct groups of macaques within cynomolgus and rhesus species that differ in their susceptibility to Mtb infection. We demonstrate significant differences in *ex vivo* iNKT frequency between groups, which trends towards an association with susceptibility to Mtb, but no significant difference in overall iNKT proliferative responses. Susceptible animals exhibited a skewed CD4⁺/CD8⁺ iNKT subset ratio in comparison to more Mtb-resistant groups. Correlation of iNKT subsets post BCG vaccination with clinical disease manifestations following Mtb challenge in the Chinese cynomolgus and Indian rhesus macaques identified a consistent trend linking increased CD8⁺ iNKTs with favourable disease

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outcome. Finally, a similar iNKT profile was conferred by BCG vaccination in rhesus macaques. Our study provides the first detailed characterisation of iNKT cells in macaque tuberculosis infection, suggesting that iNKT repertoire differences may impact on disease outcome, which warrants further investigation.

Keywords

tuberculosis; correlates of protection; macaque; iNKT; CD1d

1 Introduction

Tuberculosis (TB), caused by *Mycobacterium tuberculosis* (Mtb), is an ongoing pandemic due to imperfect diagnostic tools, rising drug resistance and lack of an effective vaccine [1, 2]. Currently, Mtb causes the highest number of deaths related to infection globally. Tuberculosis is characterised by cavitating granulomas in the lung causing transmission via respiratory secretions [3]. Failure to resolve infection is due to a complex and prolonged interaction of the pathogen with the host, involving a multifaceted immune response which to date is only incompletely understood [4]. Historically, strong pro-inflammatory immune responses generating IFN- γ and TNF- α were assumed to provide protective immunity against Mtb infection, based on studies demonstrating that complete absence of these cytokines causes exacerbated disease [5, 6]. However, there is increasing evidence that these cytokines play a more complex role in TB immunity, with an excessive Th1 response implicated in worsening pathology [7]. Importantly, there are no definitive correlates of protection against Mtb infection, due to the difficulties associated with longitudinal studies to dissect anti-mycobacterial immune responses and correlate them with disease outcome in humans [7, 8]. Therefore, studies employing well-characterised animal models that accurately reflect human disease processes are required.

The widely used mouse model of TB infection has several limitations, as mice do not develop TB latency or caseating granuloma formation, which are hallmarks of human disease [9]. In contrast, non-human primates (NHP) are remarkably similar to humans in many aspects including their anatomy, immunology and clinical manifestations of TB [10, 11]. Both rhesus and cynomolgus macaque species have been used to study Mtb infection and TB vaccine efficacy [10–14]. Importantly, animals that fall into rhesus and cynomolgus macaque species differ with regard to their genetic background and susceptibility to Mtb, a divergence also observed after challenge with other microorganisms, including human immunodeficiency virus and *Shigella* species [15–19]. Such differences may range from a strong ability to control infection in cynomolgus of Chinese or Indonesian genotype to reduced resistance in Mauritian cynomolgus [15, 20, 21]. Variations in susceptibility are likely due to immunological differences. Therefore, investigation into the adaptive and innate cellular immune processes that underpin these differences may provide novel disease correlates of protection that may ultimately help to inform the design of new vaccine candidates.

T cells are essential for a protective host immune response to Mtb [22–24], as they induce the effector antimicrobial functions of infected phagocytes. Identifying T cell-based correlates is a critical area of ongoing research within the TB field [7, 25, 26]. Invariant NKT cells (iNKTs) are innate T cells that recognise CD1d-presented lipid antigens via an invariant T cell receptor comprised of V α 24 and V β 11 in humans, and the V α 24 chain is highly conserved in macaque [27, 28]. iNKTs are critical for an effective immune response in a variety of diseases and comprise between <0.01% - 1% of T cells in the peripheral circulation in humans [29]. Despite their low numbers, iNKTs rapidly home to sites of infection and proliferate upon antigen exposure to control disease [30]. Furthermore, they exhibit anti-TB protective functions in mouse studies both *in vitro* [31] and *in vivo* [32]. Moreover, iNKT-mediated protection against Mtb infection is demonstrated by their early release of granulysin [33, 34], IFN- γ [35] and GM-CSF [31]. Indeed, iNKTs are targets for vaccine adjuvants and incorporation of α -galactosylceramide (α GC), a potent iNKT agonist, with Bacillus Calmette-Guerin (BCG) markedly augmented the priming of CD8⁺ T cells and increased vaccine efficacy in mice [36]. Furthermore, peripheral blood iNKT numbers are reduced in human TB patients with iNKTs exhibiting an activated phenotype [37, 38], indicating a role for these innate T cells in the host immune defence against TB.

Non-human primate models allow in depth exploration of immunity to Mtb while detailing disease progression and outcome [9, 11, 39]. However, studies investigating iNKTs in these well-characterised NHP models are lacking. This study aimed to determine whether iNKT-based correlates of protection against TB can be identified in this model. We studied iNKT in four distinct groups of macaques that differ in genetic background and susceptibility to Mtb infection: Chinese cynomolgus (CC), Indonesian cynomolgus (IC), Mauritian cynomolgus (MC) and Indian rhesus (RM) macaques in BCG vaccine and Mtb challenge studies.

2 Materials and Methods

Experimental animals

The animals used in this study were rhesus macaques of Indian origin (RM), Chinese cynomolgus macaques (CC), Mauritian cynomolgus macaques (MC) and Indonesian cynomolgus macaques (IC) obtained from established breeding colonies in the United Kingdom (MC, IC, RM) and China (CC). All animals were between 4 and 10 years old at the time of sample collection and were naïve to prior exposure to mycobacterial antigens (*M. tuberculosis* infection or environmental mycobacteria), demonstrated by a negative tuberculin test while in their original breeding colony and by the IFN- γ -based Primagam test kit (Biocor; CSL, Kansas, US) or screening using an ex-vivo IFN- γ ELISPOT (MabTech, Nacka, Sweden) to measure responses to mycobacterial antigens: purified protein derivative (PPD) batch RT50 (Statens Serum Institut (SSI), Copenhagen, Denmark), and 15-mer peptide pools of ESAT-6 and CFP-10 (Peptide Protein Research Ltd., Fareham, U.K.) just prior to the start of the study.

Animals were housed in compatible social groups, in accordance with the Home Office (UK) Code of Practice for the Housing and Care of Animals Used in Scientific Procedures (1989), and the National Committee for Refinement, Reduction and Replacement (NC3Rs)

Guidelines on Primate Accommodation, Care and Use, August 2006. Animals were sedated by intramuscular (IM) injection of ketamine hydrochloride (Ketaset, 100 mg/ml, Fort Dodge Animal Health Ltd, Southampton, UK; 10 mg/kg) for procedures requiring removal from their housing. None of the animals had been used previously for experimental procedures and each socially compatible group was randomly assigned to a particular study treatment. All animal procedures and study design were approved by the Public Health England, Porton Down Ethical Review Committee, and authorised under an appropriate UK Home Office project license.

BCG Vaccination

Macaques were immunised intra-dermally in the upper left arm with 100 µl BCG vaccine, Danish strain 1331 (SSI, Copenhagen, Denmark). Vaccinations were administered within one hour of vaccine reconstitution. The viability of the BCG vaccine was confirmed to be within the expected range for the batch on each occasion.

M. tuberculosis challenge strain

The Erdman K01 stock (HPA-Sept 2011) used for challenge was prepared from stocks of the Mtb Erdman strain K01 (BEI Resources, Manassas, VA, USA). A stock suspension was initially prepared from a 5 ml bacterial starter culture originally generated from colonies grown on Middlebrook 7H11 supplemented with oleic acid, albumin, dextrose and catalase (OADC) selective agar (BioMerieux, Marcy-l'Étoile, UK). A liquid batch culture was then grown to logarithmic growth phase in 7H9 medium (Sigma-Aldrich, Gillingham, UK) supplemented with 0.05% (v/v) Tween 80 (Sigma-Aldrich, UK). Aliquots were stored at -80°C. The titre of the stock suspension was determined from thawed aliquots by enumeration of colony forming units cultured onto Middlebrook 7H11 OADC selective agar.

Aerosol exposure

The methodology and apparatus used to deliver Mtb via the aerosol route was as previously described [11]. In brief, mono-dispersed bacteria in particles were generated using a 3-jet Collison nebuliser (BGI, Butler, NJ, USA) and, in conjunction with a modified Henderson apparatus [17], delivered to the nares of each sedated primate via a modified veterinary anesthesia mask. Challenge was performed on sedated animals placed within a 'head-out', plethysmography chamber (Buxco, Wilmington, NC, USA) to enable the aerosol to be delivered simultaneously with the measurement of respired volume [11, 18]. Doses of *M. tuberculosis* that had been optimised for the evaluation of vaccine efficacy in each species were used for aerosol exposure. Rhesus macaques were challenged with a median presented dose of 821 cfu (range 515 – 1368) equating to an estimated retained dose of 117 cfu (range 73 – 195). Chinese cynomolgus macaques were challenged with a median presented dose of 7684.5 cfu (range 515 – 1368) equating to an estimated retained dose of 1095 cfu (range 6746 – 9441).

Clinical procedures

Animals were monitored daily for behavioral abnormalities including depression, withdrawal from the group, aggression, and clinical changes in feeding patterns, respiration

rate and coughing. On each occasion that required blood sample collection, aerosol challenge or euthanasia, animals were weighed, rectal temperature measured and examined for abnormalities. Red blood cell (RBC) haemoglobin levels were measured using a HaemaCue haemoglobinometer (Haemacue Ltd, Dronfield, UK) to identify the presence of anemia, and erythrocyte sedimentation rates (ESR) were measured using the Sediplast system (Guest Medical, Edenbridge, UK) to detect and monitor inflammation induced by infection with Mtb.

The time of necropsy, if prior to the end of the planned study period, was determined by experienced primatology staff and based on a combination of the following adverse indicators: depression or withdrawn behavior, abnormal respiration (dyspnoea), loss of 20% of peak post-challenge weight, ESR levels elevated above normal (>20 mm), haemoglobin level below normal limits (<100 g/dL), raised temperature (>41°C) and abnormal thoracic radiograph.

Disease burden measures

Disease burden was measured using the approach described previously [40]. In brief, a post-mortem examination was performed immediately following euthanasia and pathological changes were scored using an established system based on the number and extent of lesions present in the lungs, spleen, liver, kidney and lymph nodes. Following fixation, magnetic resonance (MR) images of the lungs were collected and lesions identified based on their signal intensity and nodular morphology relative to more normal lung parenchyma. The total lung and lesion volume relative to the fixed tissue was determined using the Cavalieri method applied to MRI image stacks, and then expressed as a ratio to provide a measure of disease burden in each animal, as previously described [31,32]. Subsequently, the fixed lungs were sliced serially and lesions counted [35]. Progressors, were animals which developed progressive TB such that the disease reached levels that met humane endpoint criteria within 12 weeks of aerosol exposure to M. tuberculosis requiring the animals to be removed from further study and euthanized. 'Controllers' were animals where disease remained below humane endpoint criteria for 12 weeks after exposure to M. tuberculosis.

Cell purification

Macaque blood samples were placed in 20 ml heparinised universal tubes. Peripheral blood mononuclear cells (PBMCs) from cynomolgus blood were isolated using accuspinn tubes (Sigma-Aldrich, Gillingham, UK) and 60% Percoll (Sigma-Aldrich, Gillingham, UK). Twenty ml 1:1 blood diluted with Phosphate buffered saline (PBS) (Lonza, Slough, UK) was centrifuged at 800 relative centrifugal force (rcf), the buffy coat was then washed three times with PBS. Rhesus macaque blood was layered directly onto Ficoll-Paque (GE healthcare, Amersham, UK) after 1:1 dilution with PBS and centrifuged at 480 rcf. All macaque PBMCs were resuspended in red blood cell lysis buffer (Life Technologies, Warrington, UK) to remove red blood cell contamination.

Flow Cytometry

CD1d Tetramer— α GC-loaded CD1d/ β 2-microglobulin complexes were generated by *in vitro* refolding as previously described [41–43]. The protein monomers were then

biotinylated via an engineered BirA motif on the C-terminus of CD1d [44]. Biotinylated lipid-loaded CD1d/ β_2 -microglobulin complexes were subsequently purified by size exclusion chromatography before conjugation to PE-streptavidin (Sigma-Aldrich, Gillingham, UK) to generate fluorescent PE-conjugated α GC-CD1d tetramers [45].

iNKT staining—The following reagents were used to stain 2×10^6 PBMCs for *ex vivo* iNKT analysis: PE-conjugated CD1d- α GC tetramers, FITC anti-V α 24 (Clone C15, Beckman Coulter, High Wycombe, UK) and APC anti-CD3 (Clone SP34-2, BD Biosciences, Oxford, UK). *In vitro* α GC expanded iNKT cells were stained with the following reagents: PE-Cy7 anti-CD8 (Clone SK1, Biolegend, London, UK), PerCP-Cy5.5 anti-CD4 (Clone OKT4, Biolegend, London, UK), PE-conjugated CD1d- α GC tetramers, FITC anti-V α 24 and APC anti-CD3 to identify iNKT subsets. Propidium iodide (Sigma-Aldrich, Gillingham, UK) was added to identify live cells, before acquisition on either a FACSCalibur or a FACSARIA II (BD Biosciences, Oxford, UK). Analysis was performed using Cell Quest (version 0.3.bfab, BD Biosciences, San Jose, US) or FloJo (version 9.7.6, Treestar, Ashland, OR, US) software; and cells were gated on live, CD3⁺ lymphocytes.

Lipid antigen

Lyophilized α GC (Avanti, Zwet, Netherlands) was solubilised in vehicle (150 mM NaCl, 0.5% Tween 20; Biorad, Hemel Hempstead, UK) to 200 μ g/ml using repeated 1-min pulses of sonication and heating to 80°C.

In vitro expansion of macaque iNKTs

Macaque PBMC (4×10^6) were seeded into 24 well plates and grown in RPMI 1640 (Lonza, Slough, UK) containing: 100 IU penicillin, 100 μ g/ml streptomycin (Lonza, Slough, UK), 1% L-glutamax (ThermoFisher Scientific, Basingstoke, UK), 10% fetal bovine serum, 1% essential amino acids, 1% non-essential amino acids, 1mM sodium pyruvate (Sigma-Aldrich, Gillingham, UK), 5 μ g/ml Plasmocin (InvivoGen, San Diego, US), 2% human AB serum (Sigma-Aldrich, Gillingham, UK) and 200 μ g/ml of α GC (Avanti, Alabaster, US). Seven days later, IL-2 (400 IU/ml) (Proleukin, Novartis pharmaceuticals, UK) was added to cultures. On day 14 all cultures were stained to determine expansion of iNKT by flow cytometry.

Statistical Analysis

GraphPad Prism version 5.01 (GraphPad Software Inc., La Jolla, CA, USA) was used for statistical analysis, and *p* values ≤ 0.05 were considered statistically significant. Statistical analysis of iNKT subset proportion between animal groups were measured using chi-squared test with post hoc analysis using Bonferroni correction in the R software package. The ratios presented for CD4⁺ and CD8⁺ were determined by dividing CD4⁺ / CD8⁺ iNKT percentages within α GC-expanded PBMC. Spearman's rank correlation coefficient test was used for all correlations. Mann-Whitney *U* test was used to test for statistical significance comparing iNKT frequency and proliferation between animal groups.

3 Results

Increased *ex vivo* iNKT frequency correlates with resistance in mycobacteria-naïve macaques

To investigate the relationship between iNKTs and disease outcome in the macaque model of *Mtb* infection, we first determined peripheral blood *ex vivo* iNKT cell frequencies across four genetically distinct groups of mycobacteria-naïve macaques comprising rhesus and cynomolgus species [15, 18, 20]. We employed a highly specific strategy of iNKT identification, by flow cytometry staining of PBMC with CD1d- α GC tetramers and anti-V α 24 after gating on live, CD3⁺ T cells (Figure 1a). Staining of live CD3⁺, CD1d- α GC tet⁺, V α 24⁺ iNKTs revealed wide inter-individual differences in iNKT frequencies within each animal group ranging between <0.001% and >0.1%. *Ex vivo* iNKT numbers were significantly higher in the cynomolgus macaque of Chinese origin (CC, median=0.0143%), which have previously been shown to have the highest resistance to TB infection [15, 20, 21], compared to those of Indonesian origin (IC, median=0.0011%, $p=0.0001$), rhesus macaque (IR, median=0.0023%, $p=0.0006$) and Mauritian cynomolgus (MC, median=0.0012%, $p<0.0001$) (Figure 1b). These results associate the most resistant macaque group with the highest *ex vivo* iNKT frequency.

Skewed iNKT subset ratios associate with *Mtb* susceptibility in macaques

Next we investigated iNKT cell function by antigen-specific *in vitro* expansion. We determined the proliferative capacity of iNKTs from animals within each macaque group by *in vitro* expansion with the potent iNKT agonist α GC (Figure 2a). We found large inter-individual differences in iNKT expansion between animals of the same genetic background, ranging between <10 - >1000-fold expansion in Indonesian cynomolgus, rhesus macaque and Mauritian cynomolgus, with Chinese cynomolgus displaying the greatest variance ranging between <1 - >10000-fold expansion. We did not observe significant differences in the fold expansion of total iNKTs between the macaque groups (CC median=8.26, IC median=22.25, RM median=16.94, MC median=15.43, $p=0.25$), although Chinese cynomolgus showed a trend towards a weaker iNKT expansion overall (Figure 2b). In contrast, stratification of expanded iNKTs according to CD4 and CD8 co-receptor expression revealed statistically significant differences in the percentages of iNKT subsets (Figure 2c). Chinese cynomolgus had a significantly greater percentage of CD8⁺ iNKTs than Mauritian cynomolgus ($p=0.02$). Likewise, rhesus macaque had a significantly higher percentage of CD8⁺ iNKTs compared to Mauritian cynomolgus ($p=0.006$) (Figure 2c). Furthermore, analysis of iNKT proportions of CD4⁺, CD8⁺, double positive (DP) and double negative (DN) subsets revealed statistically significant differences between Chinese cynomolgus vs. Mauritian cynomolgus (Pearson's Chi-squared, $p=0.0002$), Indonesian cynomolgus vs. Mauritian cynomolgus ($p=0.0004$) and Indonesian cynomolgus vs. rhesus macaque ($p=0.027$) (Figure 2d), thereby highlighting iNKT repertoire differences in naïve animal groups. In addition, analysis of CD4⁺/CD8⁺ subset ratios revealed a trend towards more skewed iNKT subset ratio in TB susceptible animals (compare resistant CC=0.61 and IC=0.71 to susceptible RM=0.27 and MC=1.57). We also observed that DP iNKTs were higher in animals with increased susceptibility to *Mtb*. Taken together, our data identify a trend toward a skewed CD4⁺/CD8⁺ iNKT subset ratio following antigen-specific expansion

with disease susceptibility and therefore we investigated this observation further in challenge and vaccination studies.

Increased CD8⁺ iNKTs are observed in macaques with better disease outcomes

Having found that increased peripheral blood iNKTs and a less biased iNKT subset ratio after antigen expansion associated with TB resistance in mycobacteria-naïve macaques, we investigated whether BCG vaccination conferred an iNKT phenotype that may associate with protection against Mtb. We first studied a cohort of four Chinese cynomolgus macaques, reasoning that since vaccination in this group rendered a high ability to control infection for 26 weeks after exposure to high dose aerosol challenge (manuscript in preparation), we may uncover an iNKT associate. Analysis of samples two weeks pre- and two weeks post- BCG vaccination revealed no change in iNKT numbers and overall proliferative capacity within individuals (Supplementary Figure 1). Furthermore, our results revealed no differences in subset proportions after antigen-specific expansion (Pearson's Chi-squared, $p=0.45$ [Figure 3a]). However, correlative analysis of the percentage of CD8⁺ and CD4⁺ iNKTs post BCG with disease burden measures consistently revealed trends of an increased percentage of antigen responsive CD8⁺ iNKTs, with a more favourable disease outcome and vice versa with regard to CD4⁺ iNKTs (Figure 3b). Macaques with increased CD8⁺ iNKT numbers showed a trend towards reduced total pathology score ($r=-0.74$, $p=0.33$) and total lung score ($r=-0.88$, $p=0.25$). Despite low sample numbers, our results revealed a possible association between increased CD8⁺ iNKTs and improved disease pathology in Chinese cynomolgus.

To further investigate CD8⁺ iNKTs and disease outcome, we studied rhesus macaques that received Mtb challenge [21, 40]. Blood samples were taken two weeks before Mtb challenge from mycobacteria-naïve rhesus macaques. Comparison of *ex vivo* iNKT frequency ($p=0.23$) and *in vitro* antigen induced expansion ($p=0.36$) revealed no differences between controllers and progressors (Figure 3c). However, by subset analysis of antigen responsive iNKTs, we demonstrated a trend toward a reduced CD4⁺/CD8⁺ ratio (ratio=0.04) in animals able to control Mtb infection compared to those that developed severe disease (ratio=0.39). Consistent with this, the trend was towards higher CD4⁺ antigen responsive iNKTs in animals that developed severe disease compared to controllers and vice versa for CD8⁺ iNKTs (CD4 $p=0.15$, CD8 $p=0.21$ [Figure 3d]). Analysis of the mean subset proportions revealed statistically significant differences between controllers and progressors (Pearson's Chi-squared $p=0.0002$ [Figure 3e]). Taken together, these results revealed a consistent trend of increased numbers of CD8⁺ iNKTs in animals that have a more favourable disease outcome.

Increased CD8⁺ iNKTs in BCG-vaccinated rhesus macaques that controlled infection

Next, we hypothesised that BCG vaccination in rhesus macaques may realign iNKTs to a CD8⁺ iNKT dominant profile that may associate with protection. To investigate this, PBMC samples were taken before and after BCG vaccination [40] and iNKT profile was assessed (Figure 4). In two of three animals able to control infection, there was a dramatic decrease in CD4⁺ iNKTs and a simultaneous increase in CD8⁺ iNKTs post BCG vaccination. In addition, both animals unable to control infection had an unchanged iNKT profile after BCG

vaccination. Overall the numbers of CD4⁺ iNKTs dropped while CD8⁺ iNKTs increased (CD4 $p=0.028$, CD8 $p=0.067$ [Figure 4a]). This was confirmed by a decrease in the ratio of CD8⁺ iNKT from pre-vaccination (ratio=0.64) to post-vaccination (ratio=0.07), and represented a significant change in the overall proportion of iNKT subsets after BCG vaccination (Pearson's Chi-squared $p<0.0001$ [Figure 4b]). Furthermore, correlations of antigen responsive iNKT subsets with disease burden measures revealed a positive trend toward increased CD8⁺ iNKT subset proportions and better disease outcome. Animals with increased CD8⁺ iNKTs had decreased total pathology ($r=-0.2$, $p=0.67$), lung lesion number ($r=-0.82$, $p=0.13$) and weight loss ($r=-0.55$, $p=0.4$). One animal was a relative outlier, with a discordance between the CD8⁺ iNKTs and lung lesion ratio. In contrast, those with increased CD4⁺ iNKT had increased total pathology ($r=0.34$, $p=0.53$), lung lesion number ($r=0.3$, $p=0.68$) and weight loss ($r=0.05$, $p>0.99$) (Figure 4c). Therefore, our results suggest that BCG vaccination induces a dominant CD8⁺ iNKT phenotype, with trends to an association with better disease outcome.

4 Discussion

This study provides the first systematic investigation of innate iNKT cells as a predictive marker of disease outcome in BCG vaccination and TB challenge studies in a well-characterised cohort of rhesus and cynomolgus macaques. We have used highly specific and validated CD1d- α GC tetramers to identify iNKTs in macaques [28, 46, 47]. Our results demonstrate that an increased peripheral blood iNKT frequency, and the ratio of CD4⁺/CD8⁺ iNKT subset proportion after *in vitro* antigen expansion with α GC, shows a trend towards increased TB resistance in mycobacteria-naïve animals. Furthermore, we observed increased proportions of antigen-expanded CD8⁺ iNKTs post BCG vaccination in rhesus macaque that controlled infection. Overall, our study identifies consistent trends towards dominant CD8⁺iNKT responses and better disease outcome in macaque TB infection, but will require further corroboration due to the limitation of low sample numbers that is inherent to TB studies in NHPs.

It has been hypothesised that robust Th1 responses from CD4⁺ T cells, including polyfunctional CD4⁺ T cells secreting IFN- γ , TNF- α , and IL-2, are associated with protection in Mtb infection. However, a vaccine that induces these immunological traits has not yet been proven efficacious [5, 48, 49]. In addition, there is uncertainty about the precise relationship between IFN- γ and TNF- α expression and protection. Indeed, the lack of immune correlates that reliably measure vaccine efficacy has been a major impediment in the development of an effective TB vaccine in humans [50]. It has only recently emerged that, whilst associated with better disease outcome, there is a lack of correlation between conventional T cell responses and protection in vaccinated infants [26], and this may even be a correlate of disease risk [7]. Therefore, as an alternative line of investigation, we investigated unconventional iNKT cell subsets in a well-characterised animal model that accurately reflects human disease [14]. This is the first study to show altered iNKT subset proportion in Mtb immunity, and also provides proof of concept for using NHPs to identify new correlates of protection against mycobacterial infections.

iNKTs in the mouse model of TB have been shown capable of providing a protective immune response [51, 52]. Furthermore, recent studies have shown that iNKT cells produced GM-CSF both *in vitro* and *in vivo* in a CD1d dependent manner during Mtb infection. GM-CSF released by iNKT cells was absolutely necessary in controlling Mtb growth in infected macrophages [31, 32]. In humans, iNKT numbers are reduced in the periphery during infection and their activation *in vitro* has been shown to exhibit anti-mycobacterial activity [34, 38, 53]. Protection stems from the release of soluble effector molecules such as IFN- γ [35], GM-CSF [31] and granulysin [34], as well as through their cytotoxic capacity [32]. In addition, iNKTs exist in a poised effector state, are able to quickly home to infection sites to orchestrate an immune response, and are activated during Mtb infection [38, 51, 54]. Our data show that the most resistant macaque group has significantly higher *ex vivo* peripheral blood iNKTs. Whilst it is a possibility this association may be linked to increased genetic diversity within the more resistant animal groups [55], reduced peripheral blood iNKT cell numbers correlate with multiple human pathologies [41, 56–58]; including active TB where reduced peripheral blood iNKT numbers were suggested as a marker of disease in human cases [37]. In addition, α GC is the prototypic iNKT antigen, and α GC-mediated *in vitro* expansion as a measure of iNKT repertoire dysfunction has previously been used to correlate iNKT defects with disease outcome in human cancer, autoimmunity and viral infections [41, 59, 60]. These diverse observations support a protective role for iNKTs in human TB.

Studies from mice and humans have described CD4⁺ and CD8⁺ iNKT phenotype as precursor readouts for identifying functionally distinct subsets of iNKTs [59, 60]. However, functional characterisation of macaque iNKTs is less well defined, likely due to the semi-energetic nature of cultured macaque iNKTs [61]. Indeed, one study in Mauritian cynomolgus found that CD4⁺ and CD8⁺ iNKTs produced comparable levels of the Th1 cytokines IFN- γ and TNF- α , but only CD4⁺ iNKTs produced IL-13 [47]. Therefore, CD4⁺ iNKT cells exhibit a Th0 profile while CD8⁺ iNKTs exhibit Th1 profiles in similar fashion to human iNKT subsets. The skewing of iNKT CD4⁺/CD8⁺ subsets is a predictive measure of iNKT repertoire defects and a marker of disease in rheumatoid arthritis and HIV infection [41, 62]. In addition, iNKT subset skewing associates with differential regulation of dendritic cell and T helper cell responses [38, 41, 62, 63]. We observe trends that show greater ratios of CD8⁺ iNKTs after α GC specific expansion in animals with better disease outcome, which is consistent in two macaque species. However, defining the precise mechanisms for these observations requires further study, as we cannot rule out the possibility of a dysfunctional iNKT repertoire in animals with severe disease. Alternatively, in animals that control infection, CD4⁺ iNKT may home to site of infection post vaccination or challenge, making them undetectable in the periphery.

The inherent benefit of working with NHPs including conserved CD1d and iNKT TCR sequences [28], and the similarity with human disease, comes with the unavoidable limitation of small sample sizes and therefore the power to demonstrate statistical differences is low. Nevertheless, the trends we observed with regard to CD8⁺ iNKT subsets as potential associates of protection are worthy of further investigation, as they were consistently observed in both rhesus and cynomolgus species. Further study into the role of innate T cells in TB, which may have been underappreciated, are warranted. Importantly,

this may provide more robust correlates of protection, the absence of which currently hinders the development of novel TB vaccines. The NHP model is ideal for this purpose as mice do not express group 1 CD1 molecules. Our findings should guide future studies designed to investigate the role of the iNKT repertoire as a potential correlate of protection in macaque TB infection.

In summary, our study identifies trends that associate increased antigen-specific expansion of CD8⁺ iNKT subsets in the peripheral blood of NHP with increased protection against TB infection. Detailed functional examination of iNKT subsets in NHP and humans is required to elucidate the mechanisms underlying this potential correlation. iNKT subsets are putative correlates of protection and deserve validation in future vaccination and challenge studies to determine the relationship with TB vaccine efficacy in NHP and in human preclinical studies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. TB-resistant Chinese cynomolgus macaque have significantly increased peripheral blood iNKT frequencies relative to other macaques.

a) Gating strategy used to identify iNKTs. CD1d-αGC tetramer⁺, Vα24⁺ iNKT are pregated on live, CD3⁺ lymphocytes. b) Representative *ex vivo* flow cytometry dot plots of iNKT cells from PBMCs of four genetically distinct groups of macaque. c) Cumulative iNKT staining data from Chinese cynomolgus (CC, n=28), Indonesian cynomolgus (IC, n=25), Indian rhesus (RM, n=48) and Mauritian cynomolgus (MC, n=24); horizontal lines show medians. Relative susceptibility to Mtb of each animal group is shown as a resistance bar below the x-axis.

Figure 2. *In vitro* antigen-induced proliferative response reveals divergent expansion of iNKT subsets.

a) Representative flow cytometry dot plots of *ex vivo* iNKTs and two weeks post- (*in vitro*) α GC mediated expansion. Expanded iNKT cells were also stained for surface expression of CD4 and CD8 to determine subset proportion. Number displayed in each quadrant indicates percentage of iNKT subset within expanded iNKT population. b) iNKT fold expansion (ratio of *in vitro* expanded iNKT/*ex vivo* frequency) between macaque groups; Mauritian cynomolgus (n=13), Indonesian cynomolgus (n=24) Chinese cynomolgus (n=24) and rhesus macaque (n=42); horizontal lines show medians. c) Percentage of iNKT subsets with CD4⁺/CD8⁻, CD4⁻/CD8⁺, CD4⁺/CD8⁺ (DP) and CD4⁻/CD8⁻ (DN) co-receptor expression in α GC expanded iNKTs within mycobacteria-naïve animals. Median values are shown. d) Bar chart showing iNKT subset proportions within each animal group relative to Mtb susceptibility shown as a resistance bar below the x-axis.

Figure 3. An increased CD8⁺ iNKT profile tends to associate with lower pathology scores and better disease outcome.

α GC-mediated iNKT expansion was measured two weeks pre- and two weeks post- BCG vaccination in four Chinese cynomolgus macaques. a) Comparison of expanded iNKT subset proportions pre- and post- BCG. b) Correlative analysis of antigen expanded CD4⁺/CD8⁻ iNKTs (upper row) or CD4⁺/CD8⁺ iNKTs (lower row) with disease burden as a result of Mtb challenge. c) iNKT profile was analysed in 16 rhesus macaques that had not been vaccinated and were classified into controllers and progressors post challenge. Comparison of peripheral blood iNKT numbers (left) and α GC mediated iNKT fold expansion (right) in controllers and progressors. d) CD4⁺/CD8⁻ (left) and CD4⁺/CD8⁺ (right) iNKT profiles after *in vitro* α GC expansion in controllers and progressors. e) Bar chart showing iNKT subset proportions in controllers and progressors after α GC expansion.

Figure 4. An increased CD8⁺ iNKT proportion after BCG vaccination may associate with decreased lung pathology in TB-susceptible rhesus macaques.

Five healthy rhesus macaques were vaccinated with BCG and blood samples were taken two weeks pre- and post- vaccination. 21 weeks after BCG vaccination the animals were challenged with virulent Mtb and were stratified into progressors and controllers. a) α GC expanded CD4⁺/CD8⁻ and CD4⁻/CD8⁺ iNKTs are analysed in animals pre- and post- vaccination. Progressors (square) and controllers (triangle). b) Bar chart showing the proportions of CD4⁻/CD8⁻, CD4⁺/CD8⁻, CD4⁻/CD8⁺ and CD4⁺/CD8⁺ iNKT subsets within α GC expanded iNKT pre- and post- BCG vaccination. c) Correlative analysis of CD4⁺/CD8⁻ (upper row) and CD4⁻/CD8⁺ (lower row) iNKTs against disease burden.